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Increased Sensitivity of Histidinemic Mice to UVB Radiation Suggests a Crucial Role of Endogenous Urocanic Acid in Photoprotection

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Urocanic acid (UCA) is produced by the enzyme histidase and accumulates in the stratum corneum of the epidermis. In this study, we investigated the photoprotective role of endogenous UCA in the murine skin using histidinemic mice, in which the gene encoding histidase is mutated. Histidase was detected by immunohistochemistry in the stratum granulosum and stratum corneum of the normal murine skin but not in the histidinemic skin. The UCA content of the stratum corneum and the UVB absorption capacity of aqueous extracts from the stratum corneum were significantly reduced in histidinemic mice as compared with wild-type mice. When the shaved back skin of adult mice was irradiated with 250 mJ cm^{-2} UVB, histidinemic mice accumulated significantly more DNA damage in the form of cyclobutane pyrimidine dimers than did wild-type mice. Furthermore, UVB irradiation induced significantly higher levels of markers of apoptosis in the epidermis of histidinemic mice. Topical application of UCA reversed the UVB-photosensitive phenotype of histidinemic mice and increased UVB photoprotection of wild-type mice. Taken together, these results provide strong evidence for an important contribution of endogenous UCA to the protection of the epidermis against the damaging effects of UVB radiation.

Journal of Investigative Dermatology (2011) **131,** 188–194; doi:10.1038/jid.2010.231; published online 5 August 2010

INTRODUCTION

UVB irradiation is highly mutagenic and is considered to be the main cause of skin cancer, the most frequent malignancy in humans (Kripke and Ananthaswamy, 2003). In the epidermis, cells susceptible to malignant transformation are located in the basal layer. These cells are partially protected against the damaging effects of incident UVB by reflection of radiation at the skin surface and its absorption by chromophores in suprabasal layers. While reflection is determined by the structure of the skin surface, absorption is determined by the extinction coefficient and the spatial distribution of chromophores. Melanin, aromatic amino-acid residues of proteins, and DNA are prominent UVB chromophores of the epidermis (Young, 1997).

Urocanic acid (UCA) has been suggested to be an important UV photoprotectant as it has a high extinction coefficient in the wavelength range from 260 to 310 nm and is present in the stratum corneum (Zenisek *et al.*, 1955; Tabachnik, 1957). The ability of UCA to absorb UVB under conditions present in the outermost layer of the skin was confirmed by topical application of UCA onto the human skin, which led to an increase in photoprotection (Baden and Pathak, 1967; de Fine Olivarius *et al.*, 1996). Upon UV irradiation, the enzymatically generated *trans*-isomer of UCA is partially converted into the *cis*-isomer, which has virtually the same UV absorbance characteristics as *trans*-UCA (Kurogochi *et al.*, 1957). In addition to direct absorption of UVB radiation, UCA isomers can show protection by scavenging hydroxyl radicals that are generated by UVB irradiation (Kammeyer *et al.*, 1999, 2001). For several years, UCA was used as a component of commercial sunscreens until reports on the immunosuppressive properties of *cis*-UCA prompted the withdrawal of UCA-containing sunscreens from the market (DeFabo and Noonan, 1983; Andersen, 1995).

Endogenous UCA of the stratum corneum is produced by histidase, also known as L-histidine ammonia lyase (EC 4.3.1.3) (Gibbs *et al.*, 2008). *Hal*, the gene encoding histidase, is expressed in the liver and the epidermis (Taylor *et al.*, 1990; Eckhart *et al.*, 2008). Expression of histidase is weak or negative in proliferating keratinocytes but is strongly upregulated during differentiation of keratinocytes *in vitro* (Eckhart *et al.*, 2008). Mutations in the *Hal* gene have been identified as the cause of histidinemia, a benign metabolic disorder (Lam *et al.*, 1996). These mutations lead to an increased concentration of histidine and a decreased concentration of UCA in blood, urine, and epidermis (Baden

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Abbreviations: CPD, cyclobutane pyrimidine dimer; UCA, urocanic acid

Received 23 December 2009; revised 20 May 2010; accepted 3 June 2010; published online 5 August 2010

et al., 1969; Lam *et al.*, 1996). A spontaneous mutation (R322Q) in the *Hal* gene leading to the loss of histidase activity was detected in histidinemic mice, also known as Peruvian mice (Kacser *et al.*, 1973; Selden *et al.*, 1995). To the best of our knowledge, the sensitivity of histidinemic mice to UV-induced DNA damage has not been reported so far.

In this study, we utilized histidinemic mice to investigate the roles of histidase and UCA in the UVB protection function of the stratum corneum. Our results provide experimental support for a long proposed, yet untested, photoprotective role of epidermal UCA.

RESULTS

Histidase is strongly expressed in the stratum granulosum of wild-type mice but not in histidinemic mice

The expression pattern of histidase in the human and murine skin was determined by immunolabeling with a monoclonal antibody against a highly conserved stretch of 100 amino-acid residues at the C terminus of human histidase. Expression of histidase in the human skin was confined to the stratum granulosum and the stratum corneum (Figure 1a and b). In the stratum corneum, immunoreactivity was irregular, indicating partial masking of the epitope after cornification.

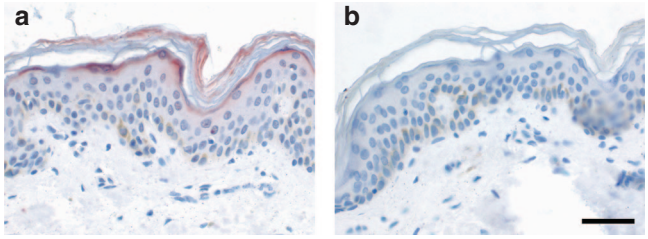

Figure 1. Histidase is expressed in the stratum granulosum of the human epidermis. (**a**) Expression of histidase in the human skin was detected by immunohistochemistry (red) using a monoclonal anti-histidase antibody. (**b**) Replacing the anti-histidase antibody with an isotype control antibody abolished the staining and confirmed the specificity of the immunodetection. The sections were counterstained with hematoxylin (blue). Bar = 50 μm.

In the murine skin, histidase was strongly expressed in the stratum granulosum of wild-type mice where it colocalized with the keratinocyte late differentiation marker loricrin (Figure 2a–c). By contrast, histidase was undetectable in the skin of histidinemic mice, although the expression pattern of loricrin was normal (Figure 2d–f). The absence of histidase protein in the histidinemic skin appeared to be caused by a post-transcriptional effect of the mutation in the *Hal* gene because histidase mRNA was expressed at equal levels in the wild-type and histidinemic skin (Supplementary Figure S1 online). Histidase was also absent in the epidermis of newborn histidinemic mice, whereas it was expressed in differentiated epidermal keratinocytes of newborn wild-type mice (not shown).

Lack of wild-type histidase is associated with a decrease in the concentration of UCA and suppression of the UVB absorption capacity of the stratum corneum

To evaluate consequences of the strongly reduced abundance, if not elimination, of the histidase protein in the epidermis of histidinemic mice, we determined concentrations of the substrate and the product of the reaction catalyzed by histidase, i.e., histidine and *trans*-UCA, respectively. Mice were shaved and the stratum corneum was prepared by tape stripping and lysis in potassium hydroxide (KOH). Histidine and *trans*-UCA were determined by high-performance liquid chromatography. As shown in Figure 3a and b, the concentration of histidine was increased >10-fold, whereas the concentration of UCA was decreased to approximately one-tenth in the stratum corneum of histidinemic mice relative to that of wild-type mice. In a separate experiment, the tape-stripped stratum corneum was extracted with phosphate-buffered saline containing the mild detergent Tween-20. The extracts were analyzed for the absorbance at a wavelength of 280 nm, which is close to the absorption maximum of UCA (Hanson and Simon, 1998). Stratum corneum extracts from histidinemic mice absorbed significantly less UV radiation than did those from wild-type mice (Figure 3c). As shown in Figure 3d, absorbance of the

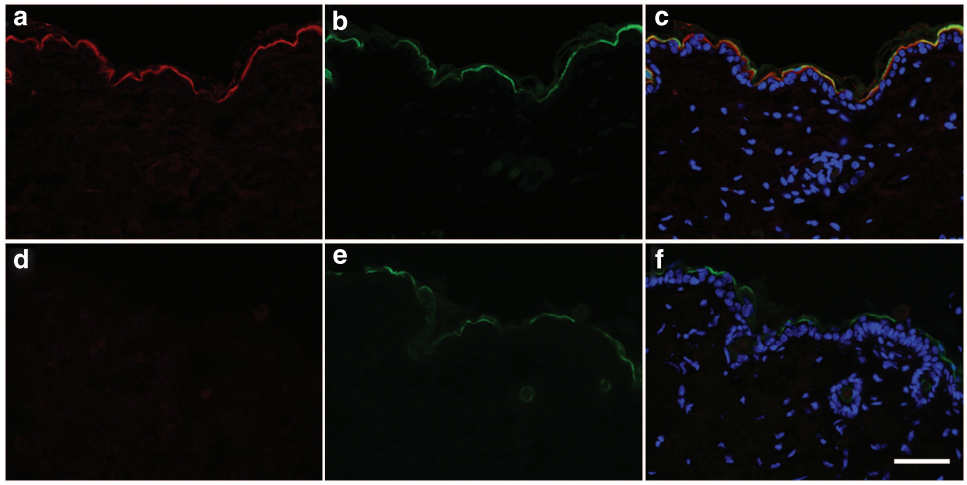

Figure 2. The epidermis of histidinemic mice lacks histidase but shows otherwise normal differentiation of keratinocytes. (**a–c**) Thin sections of the skin from wild-type mice and (**d–f**) histidinemic mice were coimmunolabeled with antibodies against (panels **a**, **d**) histidase and (panels **b**, **e**) loricrin. Panels **c** and **f** show images merged from immunofluorescence labeling of histidase (red) and loricrin (green) and DNA labeling with Hoechst dye (blue). Bar = 50 μm.

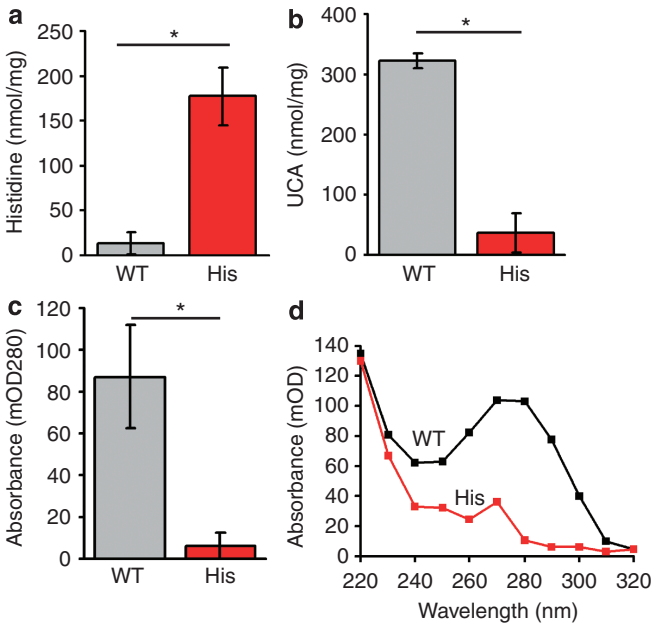

Figure 3. The stratum corneum of histidinemic mice has a decreased urocanic acid content and a decreased capacity to absorb UVB radiation. (**a**) The concentrations of histidine and (**b**) *trans*-urocanic acid (UCA) in the tape-stripped stratum corneum from the shaved back skin of wild-type (WT) and histidinemic (his) mice were determined by HPLC. (**c**) Tape strips from an adjacent site of the back of the same mice were extracted with an aqueous buffer, and the absorbance at 280 nm was determined by spectrophotometry. The bars indicate mean absorbance among each group of mice. Bars indicate the mean ± SD; $n = 5$ per group. Asterisks indicate statistically significant differences. (Panel **a**) $P = 0.0001$, (panels **b**, **c**) $P = 0.001$. mOD, milli-units of optical density. Representative absorbance spectra of these aqueous extracts from the tape-stripped stratum corneum are shown in panel **d**.

water-soluble fraction of mutant mice was smaller than that of control mice at all wavelengths of the UVB range.

Histidinemic mice display an increased sensitivity to UVB-induced DNA damage

To test the physiological relevance of UVB absorption by UCA *in situ*, the shaved back skin of mice was irradiated using a lamp that emits UVB radiation with a peak emission at 313 nm (Supplementary Figure S2 online), and the skin was investigated 24 hours later. Irradiation with 250 mJ cm^{-2} UVB caused the formation of cyclobutane pyrimidine dimers (CPDs) mostly in cells of the epidermis with some CPDs also being detectable in dermal cells (Figure 4a and b). Consistently, more nuclei containing CPDs were present in the skin of histidinemic mice than in the skin of wild-type mice (Figure 4a and b). To quantify the DNA damage, DNA was extracted from the irradiated epidermis and analyzed by an ELISA specific for CPDs. The homozygous mutation of histidase led to a 40% increase in the amount of CPDs after treatment with 250 mJ cm^{-2} UVB (Figure 4c). When a lower dose of UVB, i.e., 25 mJ cm^{-2}, was used for irradiation, the sensitivity to UVB-induced CPD formation was again significantly increased (+38%) in histidinemic mice relative to wild-type mice (Supplementary Figure S3 online), indicating that endogenous UCA has a photoprotective effect over a wide range of UVB doses.

Histidinemic mice display an increased sensitivity to UVB-induced apoptosis

DNA damage induces several cellular responses, among which programmed cell death, also known as apoptosis, is particularly well characterized (Norbury and Zhivotovsky, 2004). This cellular suicide program utilizes specific proteases such as caspase-3 to cleave a broad range of survival proteins and a specific endonuclease to cleave nuclear DNA (Enari *et al.*, 1998). In this study, we determined both the activation of caspase-3 by immunolabeling of skin sections with an antibody against the active form of caspase-3 and the breakdown of nuclear DNA by TUNEL labeling of nuclei containing DNA fragments with free 3′-OH ends. UVB induced both markers of apoptosis with a similar distribution pattern in the skin of all mice investigated (Figure 4d, e, g, and h). The frequency of epidermal cells containing active caspase-3 and the frequency of keratinocytes containing TUNEL-positive DNA fragments were significantly increased in the histidinemic skin as compared with the wild-type skin 24 hours after irradiation with 250 mJ cm^{-2} UVB (Figure 4f and i), suggesting that the increased UVB-induced damage (Figure 4c) caused a stronger cellular response in histidinemic mice than in wild-type mice.

Exogenous UCA reverses the UVB-photosensitive phenotype of histidinemic mice

To test whether the reduced amount of endogenous UCA in the histidinemic stratum corneum can be complemented by exogenous UCA as a photoprotectant, we irradiated the hairless back of newborn mice with and without previous topical application of UCA. Subsequently, DNA damage in the epidermis was determined by CPD ELISA. Like in adult mice, the mutation in histidase led to an increase in the sensitivity to UVB-mediated DNA damage when the skin was pretreated with vehicle alone. Overall, 25 mJ cm^{-2} UVB induced ~50% more CPDs in histidinemic newborn mice than in wild-type mice under these conditions (Figure 5). After application of 50 µg *trans*-UCA onto the back skin, histidinemic mice were still more sensitive to UVB-induced DNA damage than were wild-type mice treated in the same manner; however, the difference in CPD was not statistically significant. Equal amounts of exogenous UCA decreased UVB-induced DNA damage by 31% in wild-type mice and by 48% in histidinemic mice (Figure 5). Taken together, these data show that topical application of exogenous UCA improves the resistance to UVB-induced DNA damage both in the normal and the histidinemic murine skin; moreover, the protective effect of UCA is more pronounced in histidinemic mice.

DISCUSSION

A role of UCA in the sun-protection system of the skin has been the matter of a long debate (Zenisek *et al.*, 1955; Tabachnik, 1957; de Fine Olivarius *et al.*, 1996, 1998). Topical application of UCA has been shown to have a sun-protective effect; however, the role of endogenous UCA in photoprotection has been questioned (de Fine Olivarius *et al.*, 1996). In this study, we revisited the role of endogenous UCA in UVB protection of the skin. We used a well-defined model system, i.e., a genetically characterized mouse strain, and

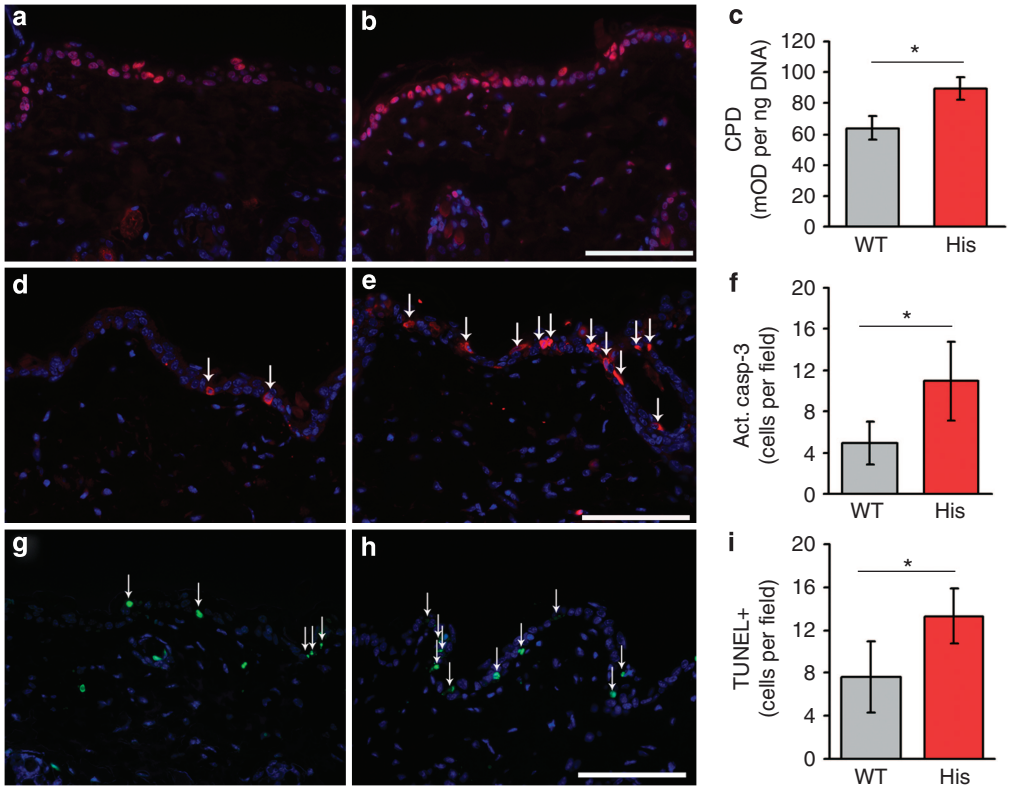

Figure 4. UVB-induced DNA damage and apoptosis in the skin of adult wild-type and histidinemic mice. Adult wild-type (**a**,**d**,**g**) and histidinemic (**b**,**e**,**h**) mice were shaved on the back and irradiated with 250 mJ cm^{-2} UVB. Twenty-four hours after the irradiation, skin samples were fixed and DNA was prepared from the epidermis. (**a**, **b**) Cyclobutane pyrimidine dimers (CPD) were immunofluorescently labeled *in situ* and (**c**) quantified in epidermal DNA preparations by ELISA. Separately, apoptotic cells were labeled by immunofluorescence with an antibody against (**d**, **e**) active caspase-3 and by (**g**, **h**) TUNEL for apoptotic DNA fragments. The frequencies of cells positive for (**f**) active caspase-3 and (**i**) TUNEL were determined under a fluorescence microscope in at least five fields of view per mouse. The bars represent the mean ± SD; $n = 5$ per group. Asterisks indicate statistically significant differences. (Panels **c**, **f**) $P<0.01$, (panel **i**) $P<0.05$. His, histidinemic; WT, wild-type.

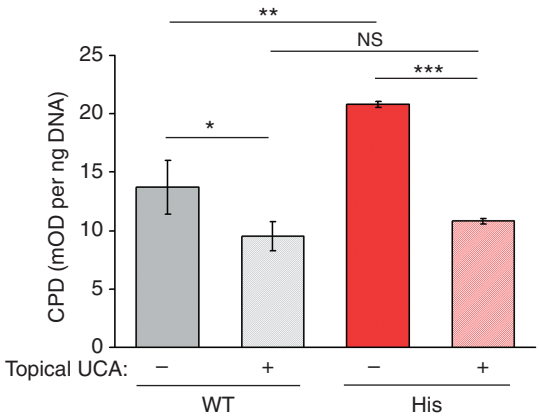

Figure 5. Topical UCA reduces the sensitivity to UVB-induced DNA damage in the murine epidermis. Overall, 50 µg UCA or vehicle (75% ethanol, 25% DMSO) were applied onto the back of newborn mice before irradiation with 25 mJ cm^{-2} UVB. The tissue was excised and frozen 1 hour after irradiation. DNA was prepared from the epidermis and the concentration of CPDs was determined by ELISA. The bars represent the mean ± SD. Wild-type (WT), topical UCA, $n = 5$; vehicle alone, $n = 4$; histidinemic (his), topical UCA, $n = 3$; vehicle alone, $n = 2$. Asterisks indicate statistically significant differences. *$P<0.05$; **$P<0.01$; ***$P<0.001$; NS, not significant. UCA, urocanic acid.

measured UVB effects that are physiologically most relevant, i.e., the formation of DNA photoproducts.

The mutation causing defective histidase activity has been mapped to a single nucleotide in the *Hal* gene (Taylor *et al.*, 1993). As mutant mice have been backcrossed for more than 20 generations into the C57BL/6 background (Mellor *et al.*, 2004), all physiological differences to normal C57BL/6 mice can be attributed unambiguously to the mutation. Previously, it was shown that the Peruvian mutation of histidase decreases the activity of the enzyme (Wright *et al.*, 1982) and its half-life in the liver (Taylor *et al.*, 1993). Our study extends the characterization of this mouse model by demonstrating normal keratinocyte differentiation as evidenced by the immunolocalization of the late keratinocyte differentiation marker loricrin. Furthermore, immunolabeling with a monoclonal antibody against histidase showed that the abundance of the histidase protein is dramatically decreased, although the level of histidase mRNA expression is normal in the histidinemic epidermis. This is comparable with the situation in the liver (Taylor *et al.*, 1993) and suggests that the mutation destabilizes the protein. The confirmation of the strong decrease in the concentration of UCA in the stratum corneum of histidinemic mice validates the model for investigation of the UVB-protective role of endogenous UCA.

Our analysis of the aqueous stratum corneum extracts demonstrates that a low concentration of *trans*-UCA is associated with a low UVB absorption capacity of the stratum corneum of histidinemic mice. Importantly, the peak absorption of the non-histidinemic stratum corneum was detected at the same wavelength as that of UCA (Hanson and Simon, 1998). Immunohistochemical and immunofluorescence analyses of the human and murine skin showed that the expression pattern of histidase is identical in both species, suggesting that histidase and its product, UCA, are regulated similarly in humans and mouse.

In the evaluation of the biological effects of UVB absorption by UCA, we focused on the direct effects of UVB on cellular DNA, i.e., the formation of photoproducts. DNA photoproducts are precursors of DNA mutations and, therefore, are intermediates in carcinogenesis. Indeed, the decrease in UCA led to a statistically significant and physiologically meaningful increase in CPD formation in histidinemic mice. As DNA damage can be partially repaired and does not necessarily disturb tissue integrity, we also investigated effects of UVB irradiation on cell physiology, i.e., activation of caspase-3 and endonuclease-mediated fragmentation of nuclear DNA. In accordance with the increased DNA damage upon UVB irradiation, histidinemic mice showed a more pronounced activation of the cell death machinery as compared with control mice. These results strongly suggest that endogenous UCA absorbs a physiologically relevant fraction of incident UVB radiation.

Although this study addressed only the first 24 hours after UVB irradiation during which the skin of normal and histidinemic mice did not undergo gross histological changes, it is possible that the sunscreening effect of UCA also reduces those long-term effects of UVB irradiation, which are mediated by DNA damage, such as malignant transformation of cells (Kripke and Ananthaswamy, 2003) and DNA photoproduct-dependent immunosuppression (Kripke *et al.*, 1992). Comparisons of normal and histidinemic mice may be useful in the investigation of these effects; however, it needs to be considered that the low level of UVB absorption by *trans*-UCA in histidinemia is also associated with decreased formation of the immunosuppressive *cis*-isomer of UCA. Therefore, the roles of both UCA isomers have to be addressed in studies of the long-term effects of UVB irradiation.

A photoprotective effect of topical UCA has previously been reported for the human skin (de Fine Olivarius *et al.*, 1996). Our results show that topical application of UCA also reduces UVB-induced DNA damage in the murine skin. Like in the published study on the human skin, the concentration of exogenous UCA in our study exceeded that of endogenous UCA. We estimate that 50 μg topical UCA covered an area of $\sim 1\,cm^2$ and that endogenous UCA was present at a concentration close to $0.75\,\mu g\,cm^{-2}$ that was previously determined in hairless mice (Gibbs *et al.*, 1993). In spite of the excess in concentration, topical UCA reduced the UVB-induced DNA damage in the wild-type mouse epidermis only by a factor of 1.44. This value is close to the protection factor of endogenous UCA, as UVB-induced DNA damage was 1.52 times higher in histidinemic mice than in wild-type mice. Both values are close to the sun protection factor of 1.58 determined for an excess of exogenous UCA on the human skin (de Fine Olivarius *et al.*, 1996). The difference in the relative photoprotective effects of endogenous and exogenous UCA remains to be investigated. It is conceivable that the pH dependence of the UV absorption characteristics of UCA (Kurogochi *et al.*, 1957) and differences in pH conditions in the various layers of the stratum corneum and on the skin surface (Fluhr *et al.*, 2004) contribute to this phenomenon. Irrespectively of the difficulty in comparing the effects of exogenous and endogenous UCA, our results show that exogenous UCA has a stronger effect in the histidinemic epidermis than in the normal epidermis (48 vs 31% reduction of UV-induced DNA damage) (Figure 5). We conclude that the UVB-photosensitive phenotype of the histidinemic mouse skin, which is caused by deficiency in endogenous UCA, can be reversed by increasing the total epidermal content of UCA.

Beyond the contribution to the basic understanding of skin physiology, this study has important implications for dermatology. Our results indicate that low concentrations of endogenous UCA may be associated with increased UV photosensitivity. The sensitivity to UVB of histidinemia patients, who have low levels of epidermal UCA, has not been investigated systematically yet. A consistently low concentration of UCA in the stratum corneum has also been reported for patients with atopic dermatitis (Kezic *et al.*, 2009). This disease is associated with deleterious mutations in the gene encoding filaggrin, the main source of the UCA precursor, histidine, in the epidermis (Scott *et al.*, 1982). Indeed, small interfering RNA-mediated knockdown of filaggrin decreases the UCA concentration and increases the UVB sensitivity of a human *in vitro* skin model (Mildner *et al.*, 2010). Moreover, it is conceivable that UCA is eluted from the skin surface during washing and bathing, which may enhance the photosensitivity of normal skin (Gers-Balag *et al.*, 1997). Therefore, human skin conditions characterized by a lower-than-normal UCA content should be investigated for their sensitivity to UVB-induced DNA damage.

In conclusion, this study establishes the critical role of histidase-catalyzed formation of endogenous UCA in photoprotection of the mammalian skin. Genetically well-defined models, such as histidinemic mice, will be instrumental in further characterization of the roles of epidermal UCA.

MATERIALS AND METHODS

Mice

Peruvian mice have been previously backcrossed into the C57BL/6 background for more than 20 generations (Mellor *et al.*, 2004). Cryopreserved embryos of these mice were obtained from the Medical Research Council (MRC; London, UK) and mice were rederived by Biomodels Austria (Vienna, Austria). Breeding was started with two pairs of homozygous mutant (histidinemic) mice and three crosses of histidinemic and C57BL/6 (wild-type) mice. Homozygous and heterozygous offspring were used for further breeding. Mice were genotyped by PCR amplification of genomic DNA from tail tips and detection of the G965A point mutation in the *Hal* gene by melting curve analysis with sequence-specific fluorescence resonance energy transfer probes according to a published protocol with modifications (Bernard *et al.*, 1998). Details of the genotyping method are provided in the Supplementary Figure S4 online. Mice were housed in a regular 12 hours light–dark cycle, and maintained at room temperature

(23 °C), with food and water available *ad libitum*. Histidinemic mice did not show gross morphological abnormalities, and their behavior appeared normal. Both female and male mice were used for irradiation experiments. All animal experiments were approved the Ethics Committee of the Medical University of Vienna.

Quantification of histidase mRNA expression

mRNA expression was quantified by real-time PCR according to a published protocol (Abtin *et al.*, 2008). Histidase cDNA was amplified with the primers Hal-forward, 5′-AGAAGCCCATGGACT GAAAC-3′ and Hal-reverse, 5′-ATGGATGTCGGTATCGAAGG-3′. The relative expression of histidase was normalized to that of the housekeeping gene β-2-microglobulin, which was amplified with the primers β2m-forward, 5′-ATTCACCCCCACTGAGACTG-3′, and β2m-reverse, 5′-TGCTATTTCTTTCTGCGTGC-3′.

Determination of histidine and UCA concentrations in the stratum corneum

The stratum corneum was prepared from the shaved back skin of mice by repeated tape stripping using D-Squame stripping discs with a diameter of 14 mm (Cuderm, Dallas, TX). The tape strips were extracted with of 0.1 M KOH following a procedure described previously (Kezic *et al.*, 2009). Four consecutive tape strips were pooled and incubated with 0.1 ml KOH and subsequently neutralized with $HClO_4$. To determine *trans*-UCA and histidine in the same high-performance liquid chromatography run, we modified the published high-performance liquid chromatography method (Kezic *et al.*, 2009) as follows: 5μl were injected onto a $250 \times 3\ mm^2$ reversed-phase Synergi Polar-RP column (Phenomenex, Utrecht, The Netherlands) using an eluent containing hydrochloric acid (6 mM), sodium octane sulfonate (0.3 mM), and acetonitrile (1%) in pure water. The retention times for histidine and *trans*-UCA were 7.02 ± 0.08 and 14.53 ± 0.05 minutes, respectively, at a flow rate of 0.4 ml per minute. The detection limits, defined as three times the signal–noise ratio, were 5.4 pmol for histidine and 2.7 pmol for *trans*-UCA. To normalize for different amounts of stratum corneum harvested by tape stripping, we determined the amount of proteins in the KOH extracts with the Micro BCA protein assay kit (Pierce, Rockford, IL) using the supplied bovine serum albumin as standard. The amount of histidine and UCA was normalized for this protein amount. Statistical significance was determined using Student's *t*-test.

Determination of UV absorption of stratum corneum extracts

Tape stripping from the murine skin was performed as described above. The stratum corneum adhering to the third to sixth tape strip from the back skin of each mouse was extracted by incubation in 50 μl phosphate-buffered saline containing 0.1% Tween-20 for 20 minutes. For each extract, UV absorbance at a wavelength of 280 nm was measured using a NanoDrop ND-1000 spectrophotometer (Peqlab Biotechnologie, Erlangen, Germany). For statistical analysis, the absorbance values at 280 nm were recorded and corrected for the absorbance caused by UV-absorbing substances extracted from the tape strip alone. Mean absorbance values of four consecutive tape strips from five mice per group were used to calculate the mean absorbance and SD of each genotype. Statistical significance was determined using Student's *t*-test.

UV irradiation of mice

The back skin of adult mice aged between 8 and 12 weeks was shaved using electric clippers and irradiated on the next day. Mice were anesthetized by intraperitoneal injection of 0.2 ml Ketalar/Rompun mix, the stock solution of which was prepared from 0.1 ml xylazin hydrochloride (2% solution, Bayer), 0.1 ml ketamin hydrochloride (100 mg ml^{-1}, Pfizer), and 1.4 ml NaCl (0.9% solution). Mice were irradiated with 25 or 250 mJ cm^{-2} of UVB using a Waldmann F15 T8 lamp (Waldmann Medizintechnik, Villingen-Schwenningen, Germany), which emits radiation with a TL12-type spectrum in the range of 285–350 nm with a peak at 313 nm. Energy output, monitored using a Waldmann UV meter, was 0.57 mW cm^{-2} at a lamp-to-target distance of 50 cm.

The photoprotective effect of exogenous UCA was investigated in newborn instead of adult mice because the hairless skin of the former did not require pretreatment (shaving) and allowed an even distribution of UCA without accumulation in hair follicles. To avoid stress to animals, they were killed before application of UCA and irradiation. UCA (USB, Cleveland, OH) was dissolved in DMSO at a concentration of 10 mg ml^{-1} and then diluted in ethanol to a final concentration of 2.5 mg ml^{-1}. Overall, 50 μg UCA in DMSO/ethanol or DMSO/ethanol vehicle was applied to the back of newborn mice from litters of wild-type and homozygous mice. Half the animals of each genotype were treated with UCA solution and the others were treated with vehicle alone. After incubation for 10 minutes in the dark, the back skin was irradiated with 25 mJ cm^{-2} UVB as described above. One hour after irradiation, the irradiated corpses were shock frozen and stored at −80 °C until further investigation.

Quantification of DNA damage

CPDs were determined quantitatively by ELISA according to a protocol recommended by MBL International Corporation (Woburn, MA) with modifications. In brief, the epidermis of the murine skin was separated from the dermis using 3.8% ammonium thiocyanate, and DNA was extracted from the epidermis. DNA samples were denatured at 100 °C for 10 minutes, rapidly chilled in an ice bath, and transferred onto a flat-bottom 96-well microtiter plate coated with 0.003% protamine sulfate. From each sample, at least 2 dilutions corresponding to DNA amounts between 5 and 100 ng per well were analyzed. After drying at 37 °C overnight, the precoated DNA was incubated with blocking buffer consisting of 0.25% skimmed milk, 0.05% Tween-20 in phosphate-buffered saline and then incubated with anti-CPD antibody (1:2,000; clone KTM53, Kamiya, Seattle, WA). After extensive washing, the plates were incubated with sheep anti-mouse IgG antibody conjugated with peroxidase (GE, Amersham, UK) at a dilution of 1:1,000. The plates were then washed and incubated with substrate solution (R&D Systems, Minneapolis, MN) for 30 minutes until the reaction was stopped by adding 50 μl 1 M H_2SO_4 to each well, and the absorbance was determined at 450 nm. Experiments, in which all values were within the linear range of the ELISA, were analyzed by Student's *t*-test for statistically significant differences between the histidinemic and control groups.

Detection of gene expression and apoptosis *in situ*

Skin specimens were fixed in phosphate-buffered 4.5% formaldehyde and embedded in paraffin. Thin sections were subjected to immunohistochemistry, immunofluorescence labeling and the TUNEL assay according to previously published protocols (Fischer *et al.*, 2005). Mouse monoclonal anti-histidase M04, clone 4F2 (1 μg ml^{-1}; Abnova, Taipei, Taiwan), rabbit anti-loricrin antiserum (2 μg ml^{-1}; Covance, Berkeley, CA), mouse anti-CPD antibody (1 μg ml^{-1};

Kamiya), and rabbit anti-active caspase-3 antibody (1:1,000; R&D Systems) were used as primary antibodies. The human abdominal skin was obtained from plastic surgery and was used in agreement with the guidelines of the Medical University of Vienna and according to the Helsinki protocol. The expression of histidase was determined by immunohistochemistry using the anti-histidase antibody (1 μg ml^{-1}). As a negative control, a mouse monoclonal immunoglobulin G antibody of unrelated specificity was used at the same concentration.

For the quantification of apoptosis, cells immunopositive for active caspase-3 were counted in the interfollicular epidermis in at least 10 fields of view at a magnification of ×400 under a fluorescence microscope. Positive cells in hair follicles and in the dermis were not included in the quantitative analysis. Cells containing TUNEL-positive DNA fragments were counted in 6–10 fields of view per sample at a magnification of ×200. Cells in the stratum granulosum of the epidermis were excluded because keratinocytes of this layer undergo TUNEL-positive cell death in the course of terminal differentiation independently of UV irradiation (Fischer *et al.*, 2005).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Florentine Calkoen, Nico LH Yau, Ramona Gmeiner, Maria Buchberger, Heidemarie Rossiter, Haiping Zhang, Barbara Lengauer, and Michael Mildner for technical support and helpful discussions. We are grateful to Harald Hoeger for expert maintenance of the mice.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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